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Award Number: DAMD17-02-1-0443

TITLE: Cooperative Interactions During Human Mammary Epithelial Cell Immortalization

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REPORT DATE: July 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Annual (1 Jul 2002 - 30 Jun 2003)

4. TITLE AND SUBTITLE
Cooperative Interactions During Human Mammary Epithelial Cell Immortalization

5. FUNDING NUMBERS
DAMD17-02-1-0443

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
Our laboratory has pioneered the development and use of cultured human mammary epithelial cells (HMEC) to gain information on the defects in growth control processes that allow finite lifespan HMEC to overcome all senescence barriers, reactivate telomerase, and gain immortal potential. We hypothesize that, due to the stringency of telomerase repression in humans, attaining these defects may be a key rate-limiting step in human carcinogenesis. The goal of this project has been to define the minimum number of genetic and epigenetic changes that permit telomerase reactivation and immortal transformation of finite lifespan HMEC, in a manner that models changes observed in breast cancers in vivo. During the past year, we were able to obtain immortalized HMEC using a combination of two oncogenes (c-myc and ZNF217) with pathological relevance to human breast cancer. Comparative genomic hybridization (CGH) analyses of two immortal populations obtained using c-myc and ZNF217 did not show any detectable additional changes in gene copy numbers, suggesting that along with unknown epigenetic changes, over-expression of these 2 genes together might be sufficient for immortalization. Better understanding of the underlying molecular changes involved in telomerase reactivation may provide novel prevention strategies and/or targets for therapeutic intervention in breast cancer pathogenesis.

14. SUBJECT TERMS
Breast cancer

15. NUMBER OF PAGES
12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Immortality allows the progeny of a single cell to accumulate the multiple errors needed to gain invasive and metastatic properties. Long-lived animals such as humans have developed extremely stringent mechanisms of cellular replicative senescence to prevent immortal transformation, presumably as a tumor-suppressor mechanism. Many recent studies suggest that one pathway by which stringent senescence is enforced in normal human cells is through repression of hTERT expression and telomerase activity. Normal human breast tissues show no telomerase activity, while almost all breast cancers express hTERT and show high levels of telomerase activity. Our laboratory has pioneered the development and use of cultured human mammary epithelial cells (HMEC) to gain information on the defects in growth control processes that allow finite lifespan HMEC to overcome all senescence barriers, reactivate telomerase, and gain immortal potential. We hypothesize that, due to the stringency of telomerase repression in humans, attaining these defects may be a key rate-limiting step in human carcinogenesis. Our previous work had demonstrated that cultured HMEC could be immortally transformed following exposure to combinations of *pathologically relevant* oncogenic agents. However, all of these studies had generated immortal lines containing unknown errors. In this grant, we proposed that the telomere-length based senescence barrier could be overcome by reactivation of hTERT, which required the cells to undergo changes in multiple distinct pathways. Our previous studies had suggested specific defined defects that might be involved. The goal of this project has been to define the minimum number of these genetic and epigenetic changes that permit hTERT reactivation and immortal transformation of finite lifespan HMEC, *in a manner that models changes observed in breast cancers in vivo.*

BODY

Task 1. Perform a semi-quantitative assessment of the efficiency of HMEC immortalization and associated changes in phenotype by GSE22, c-*myc*, and *ZNF217* alone or in combination.

Our previous data indicated that multiple alterations are required for p16(-) HMEC to overcome a telomere length based barrier (termed agonescence in p53(+) cells; crisis in p53(-) cells). We have postulated that the genomic instability induced by telomere dysfunction when telomeres become critically short produced errors that complimented pre-existing errors to cause reactivation of telomerase activity. The pre-existing errors in these populations resulted from exposing the HMEC to a chemical carcinogen (benzo(a)pyrene) and/or overexpression of the oncogenes c-*myc* or *ZNF217*, and/or inhibition of p53 function by the genetic suppressor element GSE22. We now wanted to use only combinations of defined errors to see if we could produce immortal transformation without the need for the undefined errors induced by telomere dysfunction. Our working hypothesis was that cells would require changes in a minimum of three distinct pathways, resulting in: (a) the hTERT gene rendered accessible to transactivation; (b) appropriate transcriptional activator(s) of hTERT being aberrantly expressed; and (c) molecules that inhibit telomerase activity being functionally eliminated.

Post-selection p16(-) HMEC from specimen 184 have no known existing genetic defects, although they have incurred spontaneous methylation of the p16 promoter. These cells have been transduced with retroviral vectors containing c-*myc*, *ZNF217*, and GSE22, alone or in combination. We have then monitored 3 lineages of each condition independently to control for jackpot effects and overgrowth by rare variants within a population. Resultant populations have been, or are in the process of being assayed for expression of the transgene; hTERT mRNA; telomerase activity, SA-βgal, and c-*myc*. They are also being assayed for morphology and growth rates; colony forming efficiency and labeling index ± TGFβ; genome copy number changes (by CGH); mean TRF length; and the number of foci of growing cells that appear when most cells have ceased growth at agonescence/crisis. In addition, we are preparing to perform Southern analysis of retroviral integration sites to assess the number of clonal sublines in our cell populations prior to and immediately after immortalization.

Our initial studies were hampered at first by the need to address a problem with c-*myc*-induced apoptosis. We had initially used the retroviral construct LXSN-c-*myc* for our studies on the effect of c-*myc* transduction alone, and had observed low levels of apoptosis using this construct. We subsequently tried to use an inducible

myc:ER fusion construct in order to be able to manipulate *myc* expression at later stages of immortalization. However, using 10-100 nM of 4-HT as inducer frequently, although not consistently, resulted in initially high levels of apoptosis, followed by nearly complete cell death in the populations prior to encountering the telomere length barrier. We have been adjusting the conditions for use of the *myc*:ER construct to enable us to use this successfully in future experiments. However, in the meantime, we have gone back to use of a non-inducible vector for *myc* transduction.

In 2 preliminary experiments in which we transduced both *ZNF217* and *myc*:ER into 184 HMEC, we observed what appeared to be uniform immortalization. CGH analyses of the immortal populations did not show any detectable changes in gene copy numbers. These results suggested that overexpression of these 2 genes might be sufficient for immortalization. In separate, NIH-supported experiments, we also demonstrated that although newly immortal p53(+) HMEC lines were capable of expressing telomerase activity, the functional p53 in these cells was able to repress this activity until telomeres became critically short and a conversion process was initiated (1).

In experiments currently ongoing, we have transduced 184 HMEC at passages 7-8 with combinations of *ZNF217*, c-*myc*, and GSE22. Three independent lineages were tracked for each combination. A summary of the assays performed and cells frozen at different passages following retroviral infections is presented in **Table I**. Growth curves are presented in **Fig.1**. The combination of overexpressed *ZNF217* and c-*myc* (ZM) produced homogeneously growing immortalized cells in 1 lineage, heterogeneously growing immortalized cells in a second lineage, and no immortalized cultures in the remaining lineage. An example of immortal HMEC in a ZM culture showing active growth compared to senescing cells in an LM culture at the same passage is presented in **Fig.2**. *ZNF217* alone (ZB) produced sporadic immortality in 1/3 lineages. No immortal clones arose in any of the three *myc* alone (LM) lineages. Observation of the ZM1 lineage that didn't immortalize showed that it initially grew better than control cells, and had detectable telomerase activity at passage 10, though less than that seen in the ZM3 lineage which showed homogeneous immortalization. We have hypothesized that the variable results obtained in the ZM experiments could be due to: (a) epigenetic influences; (b) imbalances in the levels of *ZNF217* and c-*myc* expression achieved due to the random nature of viral integrations; (c) stochastic genetic events. To address these possibilities, we are currently exploring whether: (1) a threshold level of telomerase activity might be required for these HMEC to immortalize; (2) levels of c-myc expression differ in lineages which do or do not immortalize; and (3) the p53 present in these cells could be repressing telomerase activity, precluding sufficient activity levels necessary for immortalization. To address the latter possibility, we have since transduced GSE22 into the ZM1 lineage as well as into ZB and LM lineages to determine whether inactivation of p53 affects the immortalization efficiencies of these populations. This experiment is currently in progress. A summary of the assays performed and cells frozen at different passages following retroviral infections is presented in **Table II**. Thus far we have seen that ZM1 plus GSE22 shows as much TRAP activity at passage 10 as the ZM3 population. The ZB and LM populations plus GSE22 now appear to be senescing, while the ZM1+GSE22 population appears to be giving rise to immortal cells, although still not uniformly.

We now need to assay the immortalized lineages further to determine whether or not they have arisen from selected cells, and whether they have accumulated genetic changes in addition to the transduced genes. C-*myc* and hTERT expression will be compared in lineages that have or have not undergone immortalization. In addition, we will perform Southern analyses of retroviral integration sites to assess whether or not the immortalized populations have undergone selection for clones with particular traits. Finally, we will perform CGH to determine whether the immortalized populations have accumulated additional genetic changes.

Technical Objective 2: Perform differential screening of high density cDNA microarrays to identify genes whose expression is altered in closely related finite lifespan, EL, and immortal HMEC:

This objective is slated for years 2-3 of the project.

Technical Objective 3: Use random homozygous knockout (RHKO) selection method to identify genes that suppress HMEC immortalization.

In collaboration with Dr. Stanley N. Cohen, we have employed his random homozygous knockout (RHKO) selection strategy to try to identify unknown genes whose inactivation promotes immortalization of post-selection p16(-) HMEC. In three independent experiments, a total of 32 plates were infected with both a tTA vector encoding a doxycycline-sensitive transactivator and an RHKO vector encoding a tTA responsive antisense promoter. In the first experiment, 1/9 plates containing benzo[a]pyrene-treated extended-life184Aa cells yielded a clonal outgrowth with additional proliferative potential (**Fig.3**). Unfortunately, this outgrowth ultimately senesced before sufficient material could be obtained for further analysis. In a subsequent experiment, post-selection 184 cells were infected with GSE22 to inactivate p53 function prior to infection with the RHKO vector. Despite this modification, all the RHKO-infected cells on 11 plates ultimately underwent crisis, yielding no clones with additional proliferative potential. In the third experiment, we attempted to take advantage of the differential sensitivity of finite life span and immortalized HMEC to *raf* oncogene-induced growth arrest (2) to identify immortalized cells in RHKO-infected cultures of finite life span specimen 48RS. We found unexpectedly that the *raf*-induced growth arrest was not stringent – all 12 plates infected with RHKO and tamoxifen inducible *raf-ER* constructs yielded slowly growing *raf*-resistant cells which ultimately senesced. We are currently re-evaluating the RHKO methodology before attempting any additional experiments.

KEY RESEARCH ACCOMPLISHMENTS

• Immortalized HMEC were obtained using a combination of two oncogenes (c-*myc* and *ZNF217*) with pathological relevance to human breast cancer.

• CGH analyses of two immortal populations obtained using c-*myc* and *ZNF217* did not show any detectable changes in gene copy numbers, suggesting that along with unknown epigenetic changes, over-expression of these 2 genes together might be sufficient for immortalization.

REPORTABLE OUTCOMES

A Model for Human Mammary Epithelial Cell (HMEC) Senescence and Immortalization in Vitro
Martha Stampfer, James Garbe, Charles Holst, Thea Tlsty, and Paul Yaswen, Lawrence Berkeley National Laboratory, Berkeley, CA, and UC San Francisco, San Francisco CA, presented at the *AACR Special Conference on the Role of Telomeres and Telomerase* in Cancer Dec. 7-11, 2002 in San Francisco, CA

Upregulation of TRF2 during Human Mammary Epithelial Cell Immortalization
Paul Yaswen, Tarlochan Nijjar, Jim Garbe, and Martha Stampfer, Dept. of Cell and Molecular Biology, Lawrence Berkeley National Laboratory, Berkeley, CA, presented at the *AACR Special Conference on the Role of Telomeres and Telomerase* in Cancer Dec. 7-11, 2002 in San Francisco, CA

Overcoming Telomere-based Senescence and Genomic Instability during Human Mammary Epithelial Cell Immortalization
Martha R Stampfer, Tarlochan Nijjar, James Garbe, Sandy DeVries, Fred Waldman, Paul Yaswen, Lawrence Berkeley National Lab., Berkeley, CA; U.C. San Francisco, San Francisco, CA., presented at the *94th AACR Annual Meeting* July 11-14, 2003 in Washington, DC.

CONCLUSIONS

A high level of telomerase activity is one of the most common distinguishing features of cancer tissues and tumor-derived immortal cell lines when compared to normal human somatic tissues and finite life span cells. In the absence of high telomerase levels, replicative senescence halts cell proliferation before all the errors necessary for invasive cancer can accrue. Our studies are designed to address the crucial question of what

errors are responsible for allowing the telomerase reactivation that transforms finite lifespan cultured HMEC to immortality, *in a manner that models changes observed in breast cancers in vivo*. We believe that understanding how telomerase is reactivated in human cells is of critical significance because; (a) overcoming senescence and attaining immortality may be rate-limiting in human carcinogenesis; (b) human and rodent cells have significant differences in telomere biology - the lack of strict telomerase repression and stringent senescence in rodent cells means that they can not model the human mechanisms. A better understanding of the underlying molecular changes involved in telomerase reactivation may provide novel prevention strategies and/or targets for therapeutic intervention in breast cancer pathogenesis.

The data generated in our first year of this grant supports our hypothesis that telomerase is repressed by multiple mechanisms in HMEC. We are now determining whether overexpression of ZNF217 and c-myc are sufficient by themselves to cause telomerase reactivation, particularly if inhibition of telomerase by p53 is alleviated. We will then be able to further explore the mechanisms by which these genes activate telomerase, and how this may relate to the errors incurred during breast cancer development *in vivo*.

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2. Olsen, C. L., Gardie, B., Yaswen, P., and Stampfer, M. R. Raf-1-induced growth arrest in human mammary epithelial cells is p16-independent and is overcome in immortal cells during conversion. Oncogene, *21:* 6328-6339, 2002.

Table I. *ZNF217/c-myc*: schedule of freezedowns and assays. (L=LXSN, B=BABE. M=Myc, Z=ZNF217, p=passage #)

	8p	9p		10p		11p		12p		13p		14p	
	samples	amps*	samples	amps	samples	a	samples	amps	samples	amps	samples	amps	samples
LB1	TRAP	12 AF522	TRAP, TRF, RNA, Prot, βgal, MeOHfix, Pictures	9 AF534	TRAP, RNA, DNA LI, prot, pellet	/	TRAP, RNA,PROT	5 AF552	TRAP, TRF, RNA, Prot, βgal, MeOHfix, Pictures, LI	6* AF567	TRAP^, RNA^	/	RNA, βgal, MeOH, Pict, LI, 2 pellets
LB2		8 AF530		5 AF542		/		5 AF556	TRAP, RNA,	5MOLD? AF569	TRAP, RNA,	/	2 pellets
LB3		12 AF531		5 AF543		/		5 AF557	TRAP, RNA,	5 AF570	TRAP, RNA,	/	1 pellet
LM1	TRAP	15 AF523	TRAP, TRF, RNA, Prot, βgal, MeOHfix, Pictures	7 AF535	TRAP, RNA, DNA LI, prot, pellet	/	TRAP, RNA,	5 AF547	TRAP, TRF, RNA, Prot, βgal, MeOHfix, Pictures, LI	5 AF558	TRAP, RNA,PROT	/	TRAP, RNA, Prot, βgal, MeOH, Pict, LI, pellet
LM2		2 AF526		8 AF538		/	PROT	5 AF550		5 AF564	TRAP, RNA,PROT	/	/
LM3		7 AF527		5 AF539		/		5 AF551		4 AF565	TRAP, RNA,PROT	/	1 pellet
ZB1	TRAP	12 AF524	TRAP, TRF, RNA, Prot, βgal, MeOHfix, Pictures	8 AF536	TRAP, RNA, DNA LI, prot, pellet	/	TRAP, RNA,	4 AF553	TRAP, TRF, RNA, Prot, βgal, MeOHfix, Pictures, LI	4MOLD? AF566		/	RNA, βgal, MeOH, Pict, LI, 2 pellets
ZB2		12 AF532	TRAP	5 AF444	TRAP, RNA	/	PROT	5 AF554	TRAP, RNA,	5* AF571	TRAP	/	1 pellet
ZB3		9 AF533	TRAP, pellet	9 AF545	TRAP, TRF RNA, Prot, DNA, LI, βgal, MeOH, pict,	/	3 pellets	5 AF555	TRAP, TRF RNA, Prot, DNA, LI, βgal, MeOH, pict,	3* AF572	TRAP^, RNA^ Pictures	/	TRAP, RNA, Prot, DNA, pict, MeOH, βgal, LI,
ZM1	TRAP	6 AF525	TRAP, TRF, RNA, Prot, βgal, MeOHfix, Pictures	6 AF537	TRAP, RNA, DNA LI	/	TRAP, RNA,PROT	5 AF546	TRAP, TRF, RNA, Prot, βgal, MeOHfix, Pictures, LI	5 AF563	TRAP, RNA,PROT	3* AF576	TRAP, RNA, Prot, βgal, MeOH, Pict, LI, pellet
ZM2		13 AF528	TRAP	5 AF540	TRAP, TRF RNA, Prot, DNA, LI, βgal, MeOH, pict,	/	TRAP, RNA, 2 pellets, pict	5 AF548 4 AF604	TRAP, TRF RNA, Prot, pellet, LI, βgal, MeoH, pict, pellet	5 AF 559	TRAP, RNA,PROT	4 AF573	TRAP, TRF RNA, Prot, DNA, LI, βgal, MeOH, pict,
ZM3		8 AF529	TRAP^	5 AF541	TRAP, TRF RNA, Prot, DNA, LI, βgal, MeOH, pict,	/	TRAP, RNA, 2 pellets, pict	4 AF549 4 AF605	TRAP, TRF RNA, Prot, pellet, LI, βgal, MeoH, pict, pellet	6 AF560 3AF612	TRAP, RNA,PROT	8 AF568	TRF, TRAP, RNA, Prot, DNA, pict, MeOH, βgal, LI, 3 PELLETS

	15p		16p		17p		18p		19p		20p		21p	22p	23p	
	amps	samples	amps	samples	amps	samples	amps	samples	amps	samples	amps	samples	samples	samples	amps	samples
ZB3	3 AF594 @4	TRAP, RNA, 2 pellets, pict	9* AF600	TRAP, RNA, DNA, pict,	/	TRAP, TRF RNA, Prot, DNA, LI,	5* AF623	/	/	/	x	TRAP, TRF RNA, Prot, DNA, LI,	p53 assay			

				pellet		βgal, MeOH, pict,						βgal, MeOH, pict,				
ZM2	/	TRAP, RNA, MeOH, βgal, LI,pict,	5* AF599	TRAP, RNA, pict, pellet	/	TRAP, TRF RNA, Prot, DNA, LI, βgal, MeOH, pict,	5* AF628				x	TRAP, TRF RNA, Prot, DNA, LI, βgal, MeOH, pict,	p53 assay			
ZM3	/	/	/	/	/	TRAP^, TRF^ RNA, Prot, DNA^, LI, βgal, MeOH, pict,	5 AF583	/	/	/	4 * AF603	TRAP, TRF RNA, Prot, DNA, LI, βgal, MeOH, pict,		p53 assay	12 AF629	/
ZM (ZAB-20-01)	/	/	/	/	/	/	/	/	/	2 pellets, DNA	/	TRAP, TRF RNA, Prot, DNA, LI, βgal, MeOH, pict,		p53 assay		
ZM1 (ZAB-21-01)	/	/	/	/	/	/	/	DNA	/	1 pellet	/	TRAP, TRF RNA, Prot, DNA, LI, βgal, MeOH, pict,	p53 assay			

Table II. *ZNF217/c-myc/GSE22*: schedule of freezedowns and assays. (G=GSE22)

	9p		10p		11p		12p		13p		14p	
	amps*	samples	amps	samples	amps	samples	amps	samples	amps	samples	amps	samples
ZBB1	/	/	/	TRAP, RNA, 2 pellets LI, βgal, pict,	5* AF608	TRAP, RNA, DNA, TRF,	/	TRAP, TRF RNA, Prot, pellet, LI, βgal, MeOH, pict,	x	TRAP, RNA		TRAP, TRF RNA, Prot, DNA, LI, βgal, MeOH, pict,
ZBG1	/	/	2 AF595 @2.5	TRAP, RNA, 2 pellets LI, βgal, pict,	10* AF602	TRAP, RNA, DNA, TRF	/	TRAP, TRF RNA, Prot, pellet, LI, βgal, MeOH, pict,	4* AF624	2 pellets	/	TRAP, TRF RNA, Prot, DNA, LI, βgal, MeOH, pict,
ZMB1	/	/	/	TRAP, RNA, 1 pellet LI, βgal, pict,	5* AF607	TRAP, RNA, DNA, TRF	/	TRAP, TRF RNA, Prot, pellet, LI, βgal, MeOH, pict,	/	TRAP, RNA		TRAP, TRF RNA, Prot, DNA, LI, βgal, MeOH, pict,
ZMG1	/	TRAP	3 AF595*	TRAP, RNA, 2 pellets LI, βgal, pict,	11* AF601	TRAP, RNA, DNA, TRF	/	TRAP, TRF RNA, Prot, pellet, LI, βgal, MeOH, pict,	5* AF621	3 pellets	/	TRAP, TRF RNA, Prot, DNA, LI, βgal, MeOH, pict,

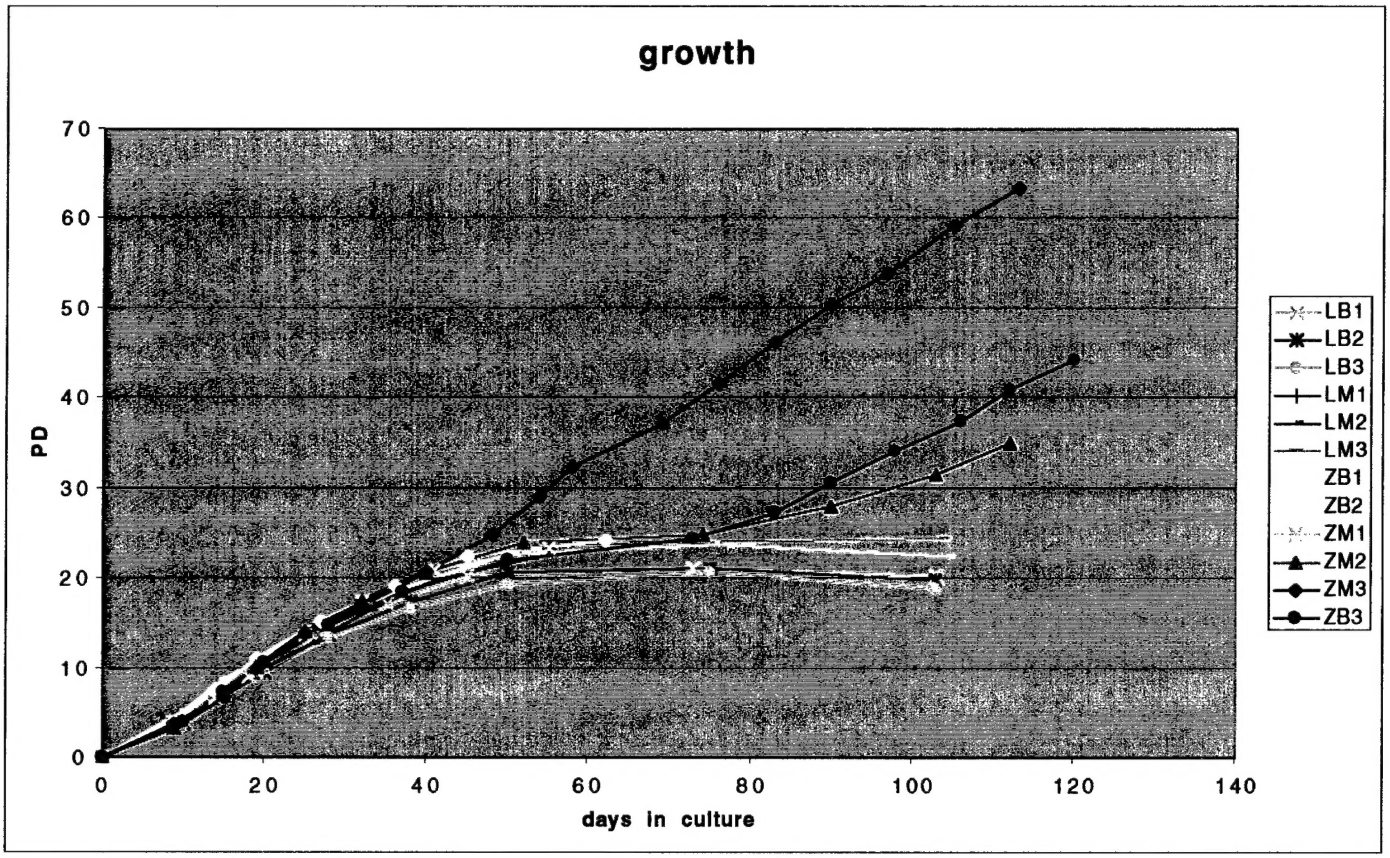

Fig.1. The cumulative population doublings of 184 cells infected with either vector alone (LXSN, L; pBABE, B), c-myc (M), and/or ZNF217 (Z) were plotted against time in days. Note that the population doublings indicated are underestimates, because they do not take plating efficiencies into account.

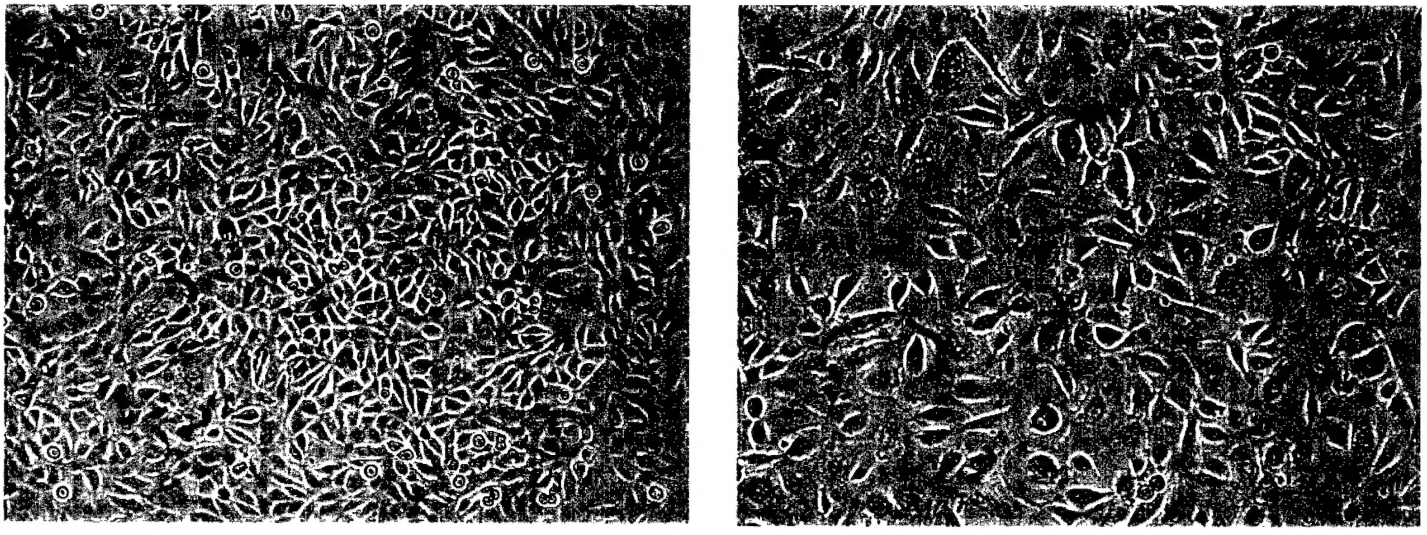

Fig.2. Area of active growth in ZM3 culture at passage 13 (A) compared to senescing cells in an LM culture at the same passage (B). Note the abundance of small cells and mitotic cells in (A), and the predominance of larger, flatter cells and the absence of mitotic cells in (B).

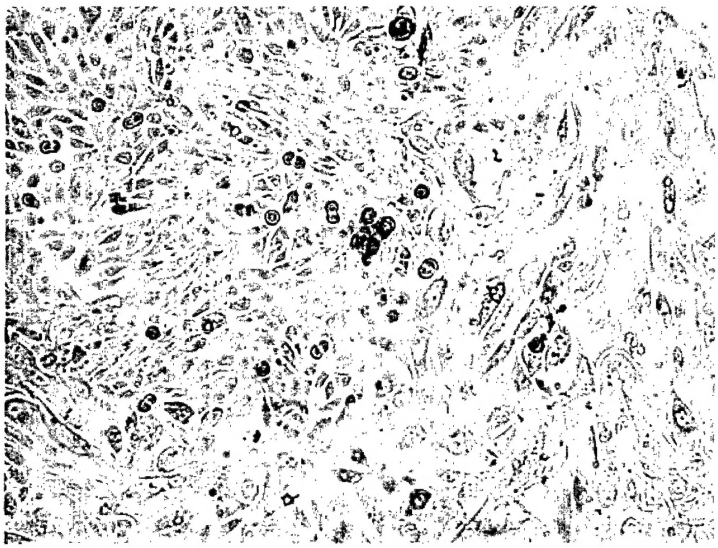

Fig.3. Area of active growth in 184Aa culture infected with RHKO virus. Note the abundance of small and mitotic cells in the upper left section of the figure compared to larger, flatter, non-mitotic cells in the lower and right sections of the figure.